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Interlaboratory Variation of Vitamin D[1]) Metabolite Measurements

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Summary: Interlaboratory variation of the measurement of 25-hydroxy vitamin D, 24,25-dihydroxy vitamin D and 1,25-dihydroxy vitamin D by six laboratories in the Netherlands was studied. Three different serum samples and two different standard solutions of each metabolite were assayed. Substantial interlaboratory variation was found for the measurement of serum samples. The mean interlaboratory CV's for the 25-hydroxy vitamin D, 24,25-dihydroxy vitamin D and 1,25-dihydroxy vitamin D assays in the three sera were 48%, 38% and 20% respectively. The measurement of standard solutions of all metabolites showed relative little variation (mean CV 8%). The small number of samples allowed no evaluation of intralaboratory variation. The much higher CV's of the measurements of serum samples, when compared to standard solutions, may be attributed to differences in extraction and purification procedures which are probably responsible for the presence of varying amounts of interfering substances during the final quantification of metabolites.

Ringversuch zur Bestimmung von Vitamin D-Metaboliten

Zusammenfassung: In einem Ringversuch wurde die Bestimmung der 25-Hydroxy-, 24,25-Dihydroxy- und 1,25-Dihydroxy-Metabolite von Vitamin D in 6 Laboratorien der Niederlande überprüft. Drei verschiedene Serumproben und zwei verschiedene Standardlösungen jedes Metaboliten wurden untersucht. Für die Bestimmung in Serumproben wurden erhebliche Unterschiede zwischen den Laboratorien festgestellt. Die mittleren Variationskoeffizienten zwischen den Laboratorien für die Bestimmung der 25-Hydroxy-, 24,25-Dihydroxy- und 1,25-Dihydroxy-Metaboliten von Vitamin D in den drei Seren betrugen 48%, 38% und 28%. Die Bestimmung in den Standardlösungen aller

[1]) The term vitamin D includes both D_2 and D_3 derivatives. However, vitamin D_2 metabolite concentrations are assumed to be negligible.

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Metaboliten zeigte einen relativ geringen Variationskoeffizienten von im Mittel 8%. Die geringe Zahl der Proben erlaubte keine Auswertung der Variation innerhalb der einzelnen Laboratorien. Verglichen mit der Bestimmung in Standardlösungen beruhen die sehr viel höheren Variationskoeffizienten der Messung von Serumproben wahrscheinlich auf Unterschieden in Extraktions- und Reinigungsverfahren, die möglicherweise verantwortlich sind für die Anwesenheit unterschiedlicher Mengen störender Substanzen während der abschließenden Quantifizierung der Metabolite.

Introduction

In the past decade clinical investigations related to vitamin D metabolism have resulted in a rapidly growing amount of data on vitamin D metabolite concentrations in human blood, both in healthy subjects and in subjects suffering from various diseases influencing vitamin D metabolism (1, 2, 3). The methods in use for the measurement of plasma and serum concentrations of vitamin D metabolites have recently been reviewed by *Seamark* and coworkers (4). Reference intervals reported in this paper for the main vitamin D metabolites 25-hydroxy vitamin D, 24R,25-dihydroxy vitamin D and 1α,25-dihydroxy vitamin D vary considerably. A possible cause for this variation may be the fact that vitamin D metabolism is regulated by exposure to sunshine, dietary intake and various parameters of calcium and phosphate metabolism, all factors that vary substantially between different populations. Moreover, recent research has revealed that some of the earlier assay methods produced erroneous values because of lack of specificity (5). To what extent however, different reference intervals are caused by either racial and environmental factors or by different assay procedures remains unclear. In order to investigate comparability of results obtained with different assay procedures a collaborative study on the measurement of 25-hydroxy vitamin D, 24R,25-dihydroxy vitamin D and 1α,25-dihydroxy vitamin D was carried out by six laboratories in the Netherlands.

Materials and Methods

Vitamin D metabolites

25-hydroxy vitamin D_3 was obtained from Duphar, Weesp, The Netherlands. 24R,25-dihydroxy vitamin D_3 and 1α,25-dihydroxy vitamin D_3 were kindly donated by Hoffmann-LaRoche, Basle, Switzerland. Purity of vitamin D metabolites was verified by straight phase HPLC on a Nucleosil 10-NO_2 column developed in n-hexane/isopropanol/water (90 + 10 + 0.35 by volume) at a flow rate of 2 ml/min.

Samples

Serum was collected from healthy subjects and pooled.

From this pool three sera were prepared: A, B and C.

Serum A was prepared by diluting a portion of the pool with a solution of 50 g/l human serum albumin, fatty acid free in 9 g/l sodium chloride to 0.73 times the original concentration.

Serum B was prepared by addition of 25-hydroxy vitamin D_3, 24R,25-dihydroxy vitamin D_3 and 1α,25-dihydroxy vitamin D_3 to a part of the pool.

Serum C was the pool without modification.

The three sera were divided into 5 ml-aliquots and stored at − 40 °C.

Two standard solutions of each metabolite were prepared in ethanol and stored at − 20 °C. All six standard solutions were in the concentration range customarily used by most laboratories.

Assay methods

A brief survey of the assay techniques used by the different laboratories is given in table 1. All laboratories measured 25-hydroxy vitamin D, five measured 24R,25-dihydroxy vitamin D and four 1α,25-dihydroxy vitamin D. Serum A, B and C were measured in duplicate by each laboratory. Sera as well as ethanolic solutions were assayed according to the standard procedure of each participant.

Results

The results of the measurements of serum samples and standard solutions are shown in table 2 A–C. For each metabolite the CV's for the standard solutions were small when compared to the CV's for the serum samples. The highest interlaboratory CV was observed for the 25-hydroxy vitamin D assays, whereas the 1α,25-dihydroxy vitamin D assays showed the lowest CV. With regard to the 25-hydroxy vitamin D assays the high CV's for the serum samples were mainly caused by the very high and very low values for all three samples found by laboratory b and d, respectively. This relative deviation in the measurements of both laboratories was not seen for the standard solutions of 25-hydroxy vitamin D. The variation for both the 24R,25-dihydroxy vitamin D and the 1α,25-dihydroxy vitamin D assays seemed to be more at random. The only outstanding fact was again the relatively low values for the serum samples measured by laboratory d (except the 1α,25-dihydroxy vitamin D concentration in serum A). When the ratios of the serum concentrations found in A and C, and in B and C are compared (tab. 3), it can be seen that the values measured for A/C with the 25-hydroxy vitamin D assays are strikingly close, despite the very high interlaboratory CV for these assays. Considerably more variation in the measured ratios was found with the 24R,25-dihydroxy vitamin D and 1α,25-dihydroxy vitamin D assays.

With regard to intralaboratory CV's the number of measurements was too small to allow a reliable assessment of this variation. In addition, each laboratory used its own standard procedure for the measurement of serum samples, which means that the number of individual competitive protein binding, RIA or UV determinations for a single serum sample varied from 1 to 3. In general however, the intralaboratory CV's of all individual measurements for serum A, B and C were less than 20% for each metabolite. The intralaboratory CV's for the standard solutions derived from the individual competitive protein binding, RIA or UV measurements were less than 10% (number of measurements varied from 1 to 4).

Tab. 1. Assay techniques
CPB = competitive protein binding DBP = vitamin D binding protein

Lab	Extraction solvent*)	25-hydroxy vitamin D		24R,25-dihydroxy vitamin D		1α,25-dihydroxy vitamin D	
		Purification	Quantification	Purification	Quantification	Purification	Quantification
a	Ether	Sephadex LH-20	Reversed phase HPLC	–	–	Sephadex LH-20, Straight phase HPLC	CPB with duodenal receptor
b	Methanol/ ether	Open silicic acid column	CPB with serum DBP	Open silicic acid column	CPB with serum DPB	–	–
c	Methylene-chloride/ methanol/water	–	CPB with serum DBP	Straight phase HPLC	CPB with serum DBP	–	–
d	Ethyl acetate/ cyclohexane and ethyl acetate/cyclo-hexane/ methanol	Straight phase HPLC	CPB with serum DBP	Straight phase HPLC	CPB with serum DBP	Straight phase HPLC	RIA
e	n-Hexane/ isopropanol/ n-butanol	Straight phase HPLC	CPB with serum DBP	Straight phase HPLC	CPB with serum DBP	Straight phase HPLC	CPB with duodenal receptor
f	Ether	Sephadex LH-20	CPB with serum DBP	Sephadex LH-20, Straight phase HPLC	CPB with serum DBP	Sephadex LH-20, Straight phase HPLC	RIA

*) for all three metabolites

Tab. 2A. Results of 25-hydroxy vitamin D measurements in nmol/l.

Lab	Serum			Standard solution	
	A	B	C	1	2
a	38	68	51	150	260
b	82	138	110	172	267
c	44	81	58	153	283
d	19	38	33	139	229
e	32	61	45	140	230
f	34	64	46	146	258
Mean:	41	75	57	150	225
SD:	21	34	27	12	21
CV:	52	45	47	8	8 (%)

Tab. 2B. Results of 24R,25-dihydroxy vitamin D measurements in nmol/l.

Lab	Serum			Standard solution	
	A	B	C	3	4
a	–	–	–	–	–
b	1.7	8.4	3.1	61	78
c	3.4	8.5	3.5	68	90
d	0.9	3.0	1.5	68	82
e	3.1	9.9	3.8	55	67
f	1.8	7.2	2.6	18*)	23*)
Mean:	2.2	7.4	2.9	63	79
SD:	1.0	2.6	0.9	6	10
CV:	48	36	31	10	12 (%)

*) Not used for calculation of mean, SD and CV

Tab. 2C. Results of 1α-25-dihydroxy vitamin D measurements in pmol/l.

Lab	Serum			Standard solution*)	
	A	B	C	5	6
a	57	152	119	2.31	3.61
b	–	–	–	–	–
c	–	–	–	–	–
d	65	80	81	2.36	3.64
e	66	160	96	2.24	3.61
f	82	171	98	2.49	4.05
Mean:	67	141	98	2.35	3.73
SD:	11	41	15	0.11	0.22
CV:	16	29	16	4	6 (%)

*) Concentrations of standard solutions in nmol/l

Discussion

As far as we know no interlaboratory comparison of the measurement of vitamin D metabolites has been described in the literature until now. The results from this study reveal that only small differences exist with regard to quantification methods used in the laboratories. The interlaboratory CV's for the ethanolic solutions were well within the range of intralaboratory CV's that are usual with competitive protein binding assays for vitamin D metabolites. The measurement of serum samples however resulted in substantially more variation. Obviously these high CV's originate from extraction and

Tab. 3 A. Concentration in serum A/concentration in serum C.

Lab.	25-hydroxy vitamin D	24R,25-dihydroxy vitamin D	1α,25-dihydroxy vitamin D
a	0.74	–	0.48
b	0.74	0.55	–
c	0.75	0.97	–
d	0.57	0.62	0.80
e	0.72	0.80	0.69
f	0.74	0.69	0.84

Tab. 3 B. Concentration in serum B/concentration in serum C.

Lab.	25-hydroxy vitamin D	24R,25-dihydroxy vitamin D	1α,25-dihydroxy vitamin D
a	1.34	–	1.28
b	1.25	2.71	–
c	1.40	2.43	–
d	1.12	1.99	0.99
e	1.37	2.60	1.68
f	1.39	2.77	1.74

purification procedures in use for the serum samples. For all assays (except the 25-hydroxy vitamin D assay of laboratory a) competitive protein binding was used for quantification of metabolites. The receptor protein for the 25-hydroxy vitamin D and 24R,25-dihydroxy vitamin D assays was in all cases the serum vitamin D binding protein. This protein however, possesses high affinity for several vitamin D metabolites (including vitamin D_2 metabolites) and in addition it is sensitive to other interfering substances in serum extracts (5). Furthermore the vitamin D metabolites with high affinity for the vitamin D binding protein migrate close together in most chromatographic systems, which makes a complete separation difficult to achieve. The use of different extraction solvents and different purification procedures may therefore result in the presence of different non-specific and specific (other vitamin D metabolites) interfering contaminants during the final quantification procedure. This may, at least in part, explain the high interlaboratory CV's for both the 25-hydroxy vitamin D and 24R,25-dihydroxy vitamin D assays. It is unlikely however that this variation was caused by vitamin D_2 metabolites, because food in the Netherlands is not fortified with vitamin D_2. This was confirmed by analysis of another serum pool of 20 healthy subjects, where displacement was only found in fractions corresponding to vitamin D_3 metabolites (6).

With regard to the 1α,25-dihydroxy vitamin D assays two competitive protein binding procedures based on the chicken duodenal 1α,25-dihydroxy vitamin D receptor, and two RIA procedures were used for quantification. The duodenal receptor offers the advantage of high specificity for 1α,25-dihydroxy vitamin D (7). So far no other vitamin D metabolite has been described that possesses equally high affinity for this receptor. Data on the cross-reactivity of the antibodies used for the RIA procedures show that they are less specific than the duodenal receptor (8, 9). 1α,25-dihydroxy vitamin D can however relatively easily be separated from interfering substances by chromatographic methods. As a result, both the higher specificity of the binding proteins and the relatively easy purification of 1α,25-dihydroxy vitamin D lead to higher overall specificity for the 1α,25-dihydroxy vitamin D assays. This was confirmed by the relatively low interlaboratory CV's.

In conclusion, the quantification methods per se showed good reproducibility in this study. The measurement of serum samples however revealed high interlaboratory CV's, especially for the 25-hydroxy vitamin D and 24R,25-dihydroxy vitamin D assays, which are probably due to different extraction and purification procedures. Standardization of these procedures or improvement of the specificity of binding proteins may result in better interlaboratory precision. Moreover reference standards and reference sera should enable a continuous evaluation and comparison of assay procedures. At the present time interlaboratory variation seems to be too high for a comparison of values from different laboratories without due consideration, especially with regard to concentrations of 25-hydroxy vitamin D and 24R,25-dihydroxy vitamin D. Besides, in countries where food is fortified with vitamin D_2, interlaboratory variation may be even larger, owing to the different properties of vitamin D_2 metabolites with regard to chromatographic mobility and affinity for the binding proteins in use (10).

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